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13. ABSTRACT *(Maximum 200 Words)*

This project is investigating the role of microtubule alterations in anoikis, with a view toward re-examining the mechanism of microtubule-targeting drugs such as taxol.

During the past year, we have uncovered a new mechanism by which cell adhesion controls apoptosis. The death receptor adaptor protein FADD (FAS-associated death domain protein) is critical for anoikis as well as death ligand (e.g., FASL) – induced apoptosis. Recently, we discovered that FADD is primarily in the nucleus of attached cells, where it is unavailable for apoptosis induction. Detachment of mammary epithelial cells from extracellular matrix, however, provokes the export of FADD from the nucleus, thus promoting apoptosis.

Our preliminary work suggests that microtubule drugs can promote FADD export, suggesting a new mechanism by which they can promote apoptosis. This is expected to have major ramifications for optimizing the use of taxol or other microtubule drugs in connection with other agents that may promote apoptosis.

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INTRODUCTION:

Taxol is one of the most effective and widely used chemotherapeutic drugs for breast cancer treatment. By targeting microtubules, it causes apoptosis through an unknown mechanism. Normally, epithelial cells that have lost contact with extracellular matrix also suffer microtubule perturbations and undergo apoptosis (a process we have named "anoikis".) This raises several questions, highly relevant to breast cancer, that are addressed by this proposal: 1. Do integrins control microtubules? 2. Do microtubules play an important role in anoikis? 3. Does perturbation of microtubules with drugs such as taxol affect anoikis? As will be explained below, most of our work so far has focussed on the third question.

BODY:

Most of our work has focussed on task 2 of the Statement of Work: "We will determine the effects of microtubulue-interacting drugs or alterations of microtubule-regulatory proteins on anoikis in mammary epithelial cells."

We found that cell adhesion modulates the response of mammary epithelial cells to death ligands substantially. In particular, detachment of MCF10a cells from extracellular matrix sensitized the cells to apoptosis induction by FAS ligand. We found that the FAS adaptor protein FADD (FAS-associated death doman protein) was sequestered in the nucleus of attached MCF10a cells, but became exported in supended cells. The nuclear export was due to the interaction of a specifically phosphorylated form of FADD with an exportin called hmsn5. A manuscript decribing these results, which is currently under review in Nature Cell Biology, is enclosed.

We found that the microtubule drugs taxol and nocodazole stimulate the export of FADD from the nucleus of attached cells, which sensitizes the cells to FASL-stimulated cell death and anoikis (N.B. FADD is involved in anoikis: **Frisch, S. M.** A role for death receptor-related, death-domain proteins in anoikis. *Curr. Biol.* 9: 1047-1049, 1999.)

We are currently exploring the pathways by which these occurs. For example, microtubule drugs are known to activate the Jun-N-Terminal kinases (JNKs), and the phosphorylation sites of FADD is a potential JNK site flanked by a possible JNK-docking site. Thus we are particularly interested in the idea that microtubule drugs stimulate nuclear export of FADD by activating JNKs to phosphorylate FADD.

KEY RESEARCH ACCOMPLISHMENTS:

In summary, the major accomplishment so far is our discovery that cell adhesion and microtubule drugs control the export of FADD from the nucleus. This may represent a novel control point for anoikis and death ligand-induced apoptosis.

REPORTABLE OUTCOMES:

Please see enclosed manuscript : R.A. Screaton and S.M. Frisch. Cell adhesion regulates FADD localization and response to FASL. Nature Cell Biol. (submitted.)

CONCLUSIONS:

Microtubule drugs such as taxol may directly induce apoptosis or sensitize cells to other apoptotic stimuli by stimulating the nuclear export of the adaptor molecule FADD.

REFERENCES:

N/A

APPENDICES:

R.A. Screaton and S.M. Frisch. Cell adhesion regulates FADD localization and response to FASL. Nature Cell Biol. (submitted)

Submitted as Brief Communication/Nature Cell Biology

Cell adhesion regulates FADD localization and response to FASL

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Interaction with extracellular matrix can protect cells against several apoptotic stimuli by promoting integrin-initiated cell survival signaling. In principle, cell-matrix contact also might protect cells against death-ligand induced apoptosis by interfering with death receptor signaling complexes. Here, we report that epithelial cell adhesion prevented the FASL-induced formation of a complex between FAS and the adaptor protein FADD, thus preventing efficient caspase-8 activation. As expected, most of the FAS was at the cell surface, yet, surprisingly, most of the FADD in matrix-attached cells was in the nucleus, suggesting that inhibition of FAS signaling was due to sequestration of FADD. In suspended cells, FADD was exported from the nucleus to the cytoplasm, and FAS-FADD complexes formed rapidly in response to FASL. Both FADD nuclear import and export required serine 194 - the major phosphorylation site of FADD - and an intact death effector domain. FADD interacted with the human homologue (hsmn5) of yeast msn5, a nuclear shuttling protein that preferentially transports phosphorylated cargo proteins. A FADD S194A mutant failed to interact with hmsn5, was retained in the cytoplasm and induced apoptosis more potently than wild-type FADD. The regulation of the cellular localization of FADD may be involved in controlling the response to death ligands.

Integrin-mediated adhesion to extracellular matrix is required for cell survival as well as cell proliferation, migration and differentiation. Loss of contact between cell surface integrin receptors and matrix elicits an apoptotic response in epithelial cells known as

anoikis[1] which attached cells counteract by activating key survival signaling molecules such as FAK, ras, ILK, Akt, ERKs, c-raf and PI3K. This suggests that integrin-mediated cell adhesion may protect cells against a variety of apoptotic stimuli, which has been demonstrated in the case of the death ligand TRAIL[2], and DNA-damaging agents[3]. Here, we explored the hypothesis that cell adhesion also might interfere with the function of death receptors by preventing the assembly of apoptosis-initiating complexes on their cytoplasmic domains[4,5]. This would implicate a direct or indirect integrin-death receptor crosstalk in the control of cell survival.

To determine whether cell-matrix adhesion could protect epithelial cells against FASL-induced apoptosis, we assayed caspase activation in the mammary epithelial cell line MCF10a (figure 1a). Cells suspended for three hours were over 11-fold more sensitive to FASL than attached cells (grown on permeable filters to facilitate exposure of the cells to ligand). Anoikis accounted for less than 10% of the caspase activation at these time points. Similar results were obtained with FASL in mouse embryro fibroblasts (figure 1a) and with the agonistic FAS antibody 2R2 in MCF10a cells (data not shown).

When stimulated with a death ligand, cell surface transmembrane death receptors (FAS, TNFR1, DR4,5) initiate apoptosis via an intracellular signaling domain on their cytoplamic tail, the death domain[5] (DD). The DD of an activated death receptor recruits the initiator caspase-8 via heterotypic DD interactions with the DD-containing adaptor proteins TRADD and/or FADD[6,7]. In addition to downstream signaling effects, the lack of caspase activation in the attached cells could have been due to inadequate FAS

expression, localization or aggregation, or alternatively, to the lack of assembly of the FAS-FADD-caspase-8 complex.

First, we found that the total cellular content of FAS mRNA and FAS protein were unaltered by cell detachment (figure 1b.) Moreover, comparable amounts of FAS were present at the cell surface of both attached and suspended cells, as determined by cell-surface biotinylation (figure 1b), indicating that FAS is properly localized irrespective of attachment. Recent reports have indicated that Fas signaling is dependent upon the ability of the receptor to cluster in the plane of the plasma membrane [8,9]; however, we observed distinctly punctate cell surface localization of FAS antibody-FAS complexes on attached MCF10a cells, indicating that FAS clustering can occur efficiently (data not shown.) Furthermore, endogenous FASL mRNA (figure 1b) and protein (data not shown) were detected neither in attached nor suspended MCF10a cells, ruling out a possible contribution to caspase activation from this source. Thus, the combined data suggested that FAS in attached cells was accessible for interaction with exogenous FASL.

Next, we tested the possibility that adhesion may promote survival of attached MCF10a cells by preventing the formation of a DISC on FAS. If this were the case, caspase-8 would fail to be recruited to the receptor - and its activation prevented - in attached cells. Immunoblot analysis of the proteolytic activation of caspase-8 confirmed this prediction (figure 2a). A lack of caspase-8 activation could result from high levels of c-FLIP, an endogenous competitor of caspase-8 recruitment and activation[10]. However, although

caspase-8 activation was readily apparent in detached cells, the levels of c-$FLIP_L$ protein remained constant (figure 2b.) (MCF10a cells do not produce detectable levels of c-$FLIP_S$.) This indicated that c-$FLIP_L$ protein is not regulated by cell detachment and that caspase-8 activation in suspended MCF10a cells occurs independently of changes in c-$FLIP_L$ levels. The expression level of FADD was also not substantially altered. However, whereas 2R2 treatment resulted in efficient recruitment of FADD to FAS in suspended cells, FADD failed to be recruited to the receptor in attached cells (figure 2c); FAS itself was immunoprecipitated from the cell surface under both conditions. These results suggested that FAS-FADD association was prevented in attached cells either by an unknown inhibitory modification of either FAS or FADD, or by sequestration of one or both partners.

To address the latter possibility, we performed immunofluorescent localization of FADD in attached, TX-100-permeabilized MCF10a cells with anti-FADD antibody. Surprisingly, most of the FADD was nuclear (figure 3a), whereas most of the FAS was at the cell surface (data not shown). This staining was blocked by recombinant FADD antigen (figure 3a), indicating the specificity of the FADD staining pattern. In addition, permeabilization of formaldehyde-fixed cells with digitonin – to selectively breach the cell membrane but not the nuclear membrane – yielded no detectable immunofluorescent signal (data not shown), confirming that most of the FADD was nuclear. Furthermore, staining MCF10a cells with HA antibodies after infection with a retroviral vector bearing a HA-tagged FADD cDNA similarly revealed a predominantly nuclear localization of HA-FADD (figure 3a.) Nuclear localization of endogenous FADD was observed in other

human cell lines, such as HT1080 fibrosarcoma cells, HaCat keratinocytes, human umbilical vein endothelial cells (HUVEC), and Caco-2 colon carcinoma cells (figure 3a.) The TNFR1 death domain adaptor protein TRADD was excluded from the nucleus.

These results suggested that a fraction of the nuclear FADD pool would be exported in response to cell detachment, permitting FADD association with FAS in FASL-stimulated cells. We attempted to visualize this export by staining detached MCF10a cells with FADD antibody, but only a background immunofluorescent signal was observed, perhaps due to extraction of FADD (25 kDa) by TX-100 during permeabilizaton (and could not be overcome by crosslinking with formaldehyde or glutaraldehyde.) Thus, we assayed for nuclear export of FADD by transfection of a reporter construct consisting of a yellow fluorescent protein (YFP)-FADD fusion protein. YFP-FADD was tagged with nuclear localization signals (NLS) from SV40 T antigen at the carboxy-terminus to prevent cytoplasmic accumulation, aggregation and toxicity of the exogenous FADD protein. In attached cells, YFP signal - irrespective of the presence of FADD coding sequence - was nearly 100% nuclear, due to the synthetic NLS (figure 3b.) Treatment of attached cells expressing YFP alone or YFP-FADD with 2R2 antibody did not increase the fraction of cells showing cytoplasmic staining. However, following 15 minutes of detachment, 21% of cells exported YFP-FADD to the cytoplasm whereas YFP alone remained in the nucleus. Deletion of the death effector domain (DED; amino acids 1-80) of FADD impaired export, indicating that export of YFP-FADD required the FADD DED. 2R2 treatment had a negligible additive effect on YFP-FADD export (23% compared with 21%). Export of this magnitude could be detected as early as 5 minutes

after detachment (data not shown), indicating that FADD export is an extremely rapid response to detachment.

FADD is phosphorylated in vivo[4] in a cell cycle dependent manner at serine 194[11,12], a site that resides in a region outside the DD at the extreme C-terminus of the protein; this phosphorylation does not affect FAS-FADD interactions[4] or any other known FADD function[11]. We examined the possibility that the phosphorylation at this site might be involved in FADD export. Interestingly, mutation of serine 194 to alanine inhibited FADD export (figure 3b), implicating this site in the export of FADD.

Signal-dependent export of nuclear proteins is mediated by a family of soluble export receptors, or exportins[13-15]. The classical exportin crm1 interacts with leucine-rich nuclear export sequences (NES.) Phosphorylation of certain cargo proteins can initiate export by inducing their interactions with other exportins or accessory proteins. For example, the yeast exportin msn5/Kap142p preferentially interacts with and mediates the export of the transcription factor PHO4[16] that has been phosphorylated at multiple sites in response to environmental cues[17]; the nuclear receptor NGFI-B is exported from the nucleus in response to phosphorylation at serine 105, which lies outside its leucine–rich NESs[18].

As mutation of the serine 194 phosphorylation site of FADD prevented export in suspended cells, we hypothesized that FADD may be exported by the human homologue of msn5 (hmsn5) in response to detachment. The ability of FADD to interact with hmsn5

was assayed by cotransfection of a glutathione-S-transferase (GST)–FADD fusion protein (which contains both unphosphorylated and phosphorylated FADD) and 6XHIS-tagged hmsn5 into 293T cells (figure 4a). The wild-type FADD interacted efficiently with hmsn5, whereas the S194A mutant, the DED deletion mutant (amino acids Δ1-80), and the C-terminal tail deletion mutant (amino acids Δ193-208) failed to interact. These results correlate well with the observed sequence requirements for nuclear export. To confirm the involvement of FADD phosphorylation in its interaction with hmsn5, a maltose binding protein (MBP)-hmsn5 fusion protein was precipitated from transfected cell lysates. Western blot analysis showed preferential association of a slower-migrating form of FADD in the precipitates (figure 4a.) The form of FADD interacting with hmsn5 in in this blot appears to be multiply phosphorylated in a serine 194-dependent manner (data not shown), which is currently being analyzed. These results suggest that the phosphorylation site serine 194 is necessary for complex formation with hmsn5 either directly or indirectly through an accessory cellular protein. Recombinant GST-FADD (purified from transfected 293T cells) and hmsn5-6Xhis protein purified from bacteria interacted in vitro (figure 4a). As anticipated, this interaction was not observed using gst-FADD purified from bacteria (data not shown), supporting the requirement for phosphorylation. The interaction in vitro was stimulated by the addition of activated (Q69L) Ran protein, which forms a ternary complex together with exportins, including msn5, and their cargoes[13]. These results suggested that FADD protein interacts directly with hmsn5 through both DED and C-terminal sequences, including the phosphorylation site, although indirect interactions occurring in vivo cannot be excluded.

The possibility that leucine-rich sequences present in the death effector domain of FADD (L19-L28) might mediate export through the classical exportin, crm1/exportin-1[19] was considered. Export mediated by crm1 is inhibited by the antibiotic leptomycin B[20]. However, under conditions where it inhibited the export of an HIV Rev-GFP fusion construct[19] efficiently (figure 4b), LMB did not inhibit export of FADD. Crm1did not interact with FADD substantially above background level, under conditions where the crm1 cargo PKI[19] interacted efficiently. Although the N-terminal leucine-rich region of FADD showed weak NES activity in an in vivo assay, mutation of leucines 20 and 23 to alanine did not affect FADD export (data not shown.)

It has been proposed that the nuclear pore diffusion limit increases during apoptosis[21] which might in principle promote FADD export. We consider this unlikely as YFP-FADD is exported before the onset of detectable caspase activation in our system, the cell permeable pan-caspase inhibitor zVAD-fmk(-O-me) did not inhibit YFP-FADD export (figure 4b) - even though caspase activation was completely inhibited by this compound at later time points (data not shown) - and YFP-NLS protein did not export in suspended cells (figure 3b.)

Recently msn5/Kap142p has been shown to be required for the nuclear import of replication protein A[22], indicating that msn5 can act as both an importin or exportin (or perhaps as shuttling protein.) This raised the possibility that hmsn5 might also act as an import receptor in FADD nuclear import. Interestingly, whereas most HA-FADD (wild-type) localized to the nucleus in attached cells, HA-FADD-S194A and HA-FADDΔ1-80

were predominantly cytoplasmic (figure 4c; note that this differs from the export experiments above in that these HA constructs were not fused to an NLS and were assayed in attached rather than suspended cells.) Thus, while we did not identify a classical NLS within FADD, faithful nuclear localization of FADD requires serine 194, suggesting that both FADD import and export may be attributed to a shuttling protein.

The constitutively cytoplasmic localization of the transfected FADD-S194A mutant would be predicted to overcome the block of FASL-induced cell death in attached cells, or perhaps induce apoptosis in the absence of the ligand. We attempted to test this possibility by reconstituting FADD-knockout mouse embryo fibroblasts with FADD-wt or -S194A retrovirus. Whereas the cells (ΦNX) used to package retrovirus produced high titer FADD-wt retrovirus, we were unable to generate FADD-S194A retrovirus as the packaging cells died during puromycin selection (data not shown), suggesting enhanced toxicity of the latter. To confirm this, MCF10a cells or HT1080 cells were cotransfected with a GFP expression construct reporter and HA-FADD-wt or a series of HA-FADD mutant proteins and scored for apoptosis. Indeed, FADD-S194A induced apoptosis more efficiently than FADD-wt (figure 4d). As expected, FADDΔ1-80 and FADD-F25G were non-apoptotic. Phenylalanine 25, which is required for the DED of FADD to interact with caspase-8 [23] was also required for the apoptosis induction by the S194A mutant (figure 4d), indicating that the latter was mediated by DED interactions.

In summary, these results suggest that cell adhesion to matrix can regulate the cellular localization of FADD, thus affecting the sensitivity of the cells to FASL. Export and

import of FADD appear to require similar FADD sequences, the DED and serine 194, both of which are also required for interaction with the shuttle protein hmsn5. While it is tempting to speculate that the nuclear-cytoplasmic shuttling of FADD is triggered by phosphorylation of serine 194, the present data do not rule out the possibility that phosphoserine at this position is constitutive in a large fraction of cellular FADD and that cell adhesion controls the interaction of FADD with nuclear or cytoplasmic anchor proteins, possibly through additional phosphorylation sites. In fact, detachment of the cells did not alter the fraction of serine 194-phosphorylated FADD (data not shown). Another compelling possibility is that cell adhesion controls FADD multimerization, which in turn regulates FADD's ability to interact with hmsn5 or other transport proteins, analogous to the regulation of p53 export[24]. Consistent with this, we have found that FADD-wt interacts with itself efficiently but not with FADDΔ1-80 (data not shown). Taken together, our results suggest that regulation of FADD localization by cell-matrix adhesion may represent a novel control point for apoptosis induced by FAS and possibly other FADD-recruiting death receptors[25-27], as well as for anoikis[28,29]. It will now be important to identify cell adhesion signaling pathways that regulate FADD localization.

Materials and Methods

Reagents: Antibodies were purchased from the following sources: FADD (Clone 1) from Transduction Labs; Fas (clone 2R2) from Kamiya Biochemicals and Roche; Fas (clone B10) from Santa Cruz; FLIP (clone DAVE-2 from Alexis Corp.), TRADD (clone 37 from Transduction Labs); Caspase-8 (Clone 12F5 from Alexis Corp); anti-tetra HIS from Qiagen. Anti-mouse HRP was purchased from Bio-Rad. All isotype-specific HRP-conjugated anti-mouse antibodies were from Roche. Goat anti-IgG3-rhodamine antibody for immunofluoresence with anti-Fas 2R2 clone was purchased from Southern Biotechnology Associates. DEVD-AFC and recombinant human Fas-L were from Alexis Corp. Recombinant RanQ69L was purchased from Calbiochem. The HIV Rev-GFP construct was a kind gift of B. Henderson.

Constructs: All cDNA constructs were prepared using standard molecular biological techniques and were verified by immunoblotting with protein-specific and anti-tag antibodies, as well as fluorescence where appropriate (for GFP- and YFP-fusions). All mutant constructs were verified by PCR sequencing. Expression constructs for hmsn5 were generated using the sequence of the human homologue of yeast msn5/Kap142p that has recently been deposited on GenBank (accession number AF298880) and cloned into pcDNA3.1-myc/his (Invitrogen).

Cell Culture and Transfections: MEFs, MCF10a mammary epithelial cells, and HaCat cells were cultured in DME+10%FBS + antibiotics. Media for MCF10a cells were supplemented with MEGM 'bullet pack' components (hEGF, insulin, hydrocortisone, bovine pituitary extract; Clonetics). Caco-2 were cultured in MEM + 10% FBS. HUVECs were cultured in EGM+ endothelial cell supplements (Clonetics). For suspension experiments, cell reattachment was prevented by maintaining them in 6- or 24-well low-attachment plates (Costar) in 37°C incubator for the indicated times, in 0.5 ml or 2 ml medium volumes, respectively. Cells were transfected using Fugene (Roche) according to manufacturer's instructions. Briefly, cell cultures in 6-well dishes were incubated with DNA-lipid complexes for 8-16 hours (DNA:Fugene of 1 ug:3ul per well).

Caspase activation assays: Cells were cultured in 6-well plates (Falcon) and treated before reaching confluence, unless otherwise indicated. The night before assays were performed, the cells were re-fed with fresh medium. MCF10a cells were cultured in on cell culture inserts (0.4 um pore, Falcon # 3090) fitted to 6-well plates to minimize trypsinization times. Cells were suspended with trypsin + EDTA and resuspended in fresh medium with our without 100 ng/ml recombinant human FAS-L (Alexis) or 0.5 ug/ml anti-Fas antibody (clone 2R2, Roche or Kamiya Biochemicals). For attached controls, cells on inserts were re-fed with the same medium ± ligand/antibody above and below the insert surface to ensure cell exposure to soluble factors. Cells were collected by centrifugation and rinsed with PBS prior to lysis. Active caspases were isolated from cells by two methods: two freeze-thaw cycles in hypotonic buffer [50 mM PIPES pH 7.4, 10 mM KCl, 5 mM EGTA, 2 mm MgCl, 10 uM cytochalasin B and 1 mM DTT] or by

non-ionic detergent lysis [20 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT 0.2% Triton X-100]. 10 or 20 ug of soluble protein was assayed for the ability to process caspase substrate DEVD-AFC (Calbiochem) by fluorometry at 15 second intervals over 15 min total reaction time in a 100 uL of caspase assay buffer 20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mMEDTA, 0.1% CHAPS, 10% sucrose, pH7.2). Vmax for the reactions were recorded and reported. Proteolytic activation of caspase-8 was monitored by dissolving the Triton-insoluble pellet (which contained all detectable processed forms of caspase-8; data not shown) in 1X SDS sample buffer and immunoblotting for caspase-8.

Indirect Immunofluorescence: Cells were cultured on sterile glass coverslips in 6-well plates and fixed with fresh 4% paraformaldehyde in PBS for 20 min. at ambient temperature (all subsequent steps were performed at ambient temperature). Cells were permeabilized with fixative supplemented with 0.2% TX-100 for 2 min., and reactive aldehyde groups were quenched with 3 rinses of PBS/0.1M glycine. Non-specific cross-reactivity was blocked with PBS+3% BSA for 20 min. prior to addition of primary antibodies. Primary [1 ug/ml in PBS/BSA] and secondary [rhodamine-conjugated goat (Fab')2 anti-mouse, 7 ug/ml] antibodies were added for 30 min, and rinsed extensively with PBS. Coverslips were mounted with Vectashield containing DAPI for nuclear counterstain (Vector: H-1200), and sealed with Cytoseal (VWR). For antigen blocking experiments, 2 ug of purified recombinant MBP-FADD or 20 ug of MBP-jun protein as control was used. Images were captured with CCD camera using SPOT software and processed using Photoshop (Adobe).

Cell Surface Biotinylation: Subconfluent and suspended MCF10a cells were rinsed 3 times with cold PBS and incubated with cell membrane-impermeable NHS-LC-Biotin reagent (Pierce) at 0.5 mg/ml in PBS for 30 min at RT. Suspended cells were collected by centrifugation and resuspended in 0.5 ml 50 mM Tris pH 8, 250 mM NaCl, 2 mM EDTA + protease inhibitors. TX-100 was then added to a final concentration of 0.5%. The suspensions were syringed 10 times with a 27 gauge needle and left for 10 min on ice. For attached cells on inserts, the cells were scraped directly into lysis buffer containing 0.5% TX-100. The lysates were clarified by 20 min centrifugation at 13, 000 rpm in a microcentrifuge at 4°C. 0.13 mg from the Triton-soluble fractions was precipitated with avidin-agarose (Sigma A2036) in 0.5 ml volume for 3 hours at 4°C with end-over-end mixing.

RT-PCR: Total RNA was isolated using RNEasy Kit (Qiagen) from MCF10a cells. 6 ug of RNA was reverse-transcribed using 1 ug of oligo-dT with Superscript II (Life Technologies) in 40 ul volume. The following primer pairs were used for amplification: Fas: for: 5' ATGCTGGGCATCTGGACCCT and rev: 5' GACCAAGCTTTGGATTTCATTTC; Fas-L: for: 5' ATGCAGCAGCCCTTCAATTA and rev 5': TTAGAGCTTATATAAGCCGA; FLIP-L: for: 5' ATGTCTGCTGAAGTCATCCATC and rev: 5' TGTGTAGGAGAGGATAAGTTT; FLIP-S: same for as for FLIP-L and rev: 5' TCACATGGAACAATTTCCAAG. Cycling parameters were fixed and product yields were verified to be within the linear range for each target by serial dilution of the input cDNA.

FADD Export Assay: To generate FADD-3X SV40 NLS constructs, FADD wt cDNA and the indicated mutants were subcloned into the pEYFPnuc vector (Clontech) as BspEI-XhoI fragments. Cells were transfected overnight and passaged the next morning either onto coverslips (for attached conditions), or onto dishes (for suspended conditions) and cultured for an additional 24 hrs. For suspended cells: cultures were trypsinized, collected by centrifugation and incubated in DME -EGM supplement on low attachment dishes at 37°C. After 15 min, cell suspensions were transferred to eppendorf tubes, centrifuged at 4,000 rpm for 3 min, rinsed with PBS-S [8.5% sucrose, 0.5 mM MgCl and 0.5 mM CaCl2 in PBS), and fixed in suspension with 4% paraformaldehyde/PBS-S for 20 min with gentle mixing. Fixed cells were rinsed with PBS-S and suspended directly into Vectashield containing DAPI for mounting. The medium on attached cells was also changed to DME –EGM supplement for 15 min prior to fixation. Cells were scored for the appearance of YFP signal in the nucleus only (N) or nucleus+cytoplasm (N+C) using a fluorescence microscope. N+C cells were considered to have exported YFP.

Protein Interactions: GST proteins were purified from 293T cell lysates after transient transfection with pEBG[30]-based constructs as follows: For interactions with bacterial exportins, cells were lysed with cold PBS/1% TX-100/Complete protease inhibitors minus EDTA (Roche) /2 mM DTT/10 mM sodium pyrophosphate, 10 mM β–glycerophosphate/2U/ml apyrase (Sigma) and clarified by centrifugation. GST-proteins were purified from the Triton-soluble fraction by incubation with glutathione-sepharose (Pharmacia) for 1-2 hrs at 4°C with end-over-end mixing. After 4 washes with

PBS/0.1% TX-100 + 2 mM DTT, bead-bound proteins were equilibrated with exportin binding buffer [(EBB): 150 mM KOAc, 2 mM MgOAc, 0.1% Tween-20, 14 mM beta-mercaptoethanol, and 0.5% ovalbumin]. Cells expressing MBP-hmsn5 protein were lysed directly in EBB (-ovalbumin) and collected using amylose-agarose (NEB). For in vitro interaction experiments, purified GST-FADD proteins were incubated with C-terminally His-tagged recombinant human hmsn5 in 100 ul final volume of EBB at 4°C with periodic mixing for 75 min and rinsed 3 times with EBB. A final wash in EBB –ovalbumin was done prior to direct solubilization and boiling in 1X SDS sample buffer. For cotransfection experiments, 0.5 ug of pEBG-based constructs (GST) and 0.5 ug pcDNA3.1-based constructs (6XHIS) were cotransfected and prepared as described above.

Immunoblotting: SDS-solubilized precipitates were boiled for 3 min and resolved on 4%-20% gradient gels (Novex). Proteins were transferred to 0.45 um PVDF (Millipore) membranes, blocked in 5% milk in PBS/0.1% Tween-20 (PBS-T) for 1 hr at 20°C, and incubated with primary antibodies for 1-2 hrs at 20°C or overnight at 4°C. After extensive washing with PBS-T, membranes were incubated with secondary antibodies for 45 min-2 hrs at 20°C and again rinsed. Immunoreactive species were visualized with Pierce ECL detection reagents according to manufacturer's instructions.

Cell surface FAS co-immunoprecipitation/Western blotting: MCF10a cells were grown to ~80% confluence on cell culture inserts prior to the addition of 0.5 µg/ml 2R2 (Roche) anti-FAS antibody for various indicated times to attached or suspended cells. Cells were

pelleted in an Eppendorf microfuge for 10 seconds at 6,000 rpm, washed in PBS and then lysed in 600 uL of 1% Triton X100, 50 mM Tris pH 8.0, 200 mM NaCl, 10% glycerol by syringing with a 27 gauge needle followed by rotating for 10 minutes at 4°C. Following removal of an aliquot for protein assay, bovine serum albumin was added to cleared lysates (5 mg/ml final concentration), which were then incubated with 40 uL of a 50% slurry of protein A-sepharose for 3 hours at 4°C on a wheel. Following 3 washes with BSA and one final wash without BSA, the immunoprecipitates were boiled in 1X SDS sample buffer and analyzed by immunoblotting, sequentially probing with anti-FADD antibody /anti-IgG_1–HRP secondary antibody and then anti-FAS antibody/anti-IgG_1 secondary antibody.

Figure 1. Cell detachment stimulates the response to FASL.

1a. Caspase activation is stimulated by detachment. MCF10a cells or mouse embryo fibroblasts immortalized with T-antigen (MEFs) were grown on permeable cell culture inserts and treated with FASL or without FASL (control) for the indicated times under attached or suspended conditions, followed by cell lysis and total caspase activity assays.

1b. Cell adhesion does not affect the expression or cell-surface localization of FAS. MCF10a cells treated with or without FASL under attached (A) or suspended conditions were analyzed for FAS mRNA by RT-PCR (top panel), total FAS protein by Western blotting (second panel from top), cell-surface FAS by Western blotting of avidin-precipitated, biotinylated FAS (third panel) or FASL mRNA by RT-PCR (bottom panel.)

Figure 2. Caspase-8 activation and FAS-FADD interaction are deficient in attached cells treated with FASL.

2a. Caspase-8 activation in response to FASL is stimulated by cell detachment. MCF10a cells were treated with or without FASL under attached or suspended conditions and Triton X100-insoluble fractions were assayed for caspase-8 activation by Western blotting. Note that both the 43 kDa and 18 kDa species are enzymatically active, in that the ~10 kDa cleavage product is associated with both in vivo (M. Peter, personal comm.)

2b. Detachment does not induce c-FLIP or FADD expression. MCF10a cells were treated with or without FASL under attached (A) or suspended (S) conditions and assayed for c-FLIP$_s$ mRNA by RT-PCR (top), c-FLIP$_L$ protein by Western blotting (middle panel) or FADD protein by Western blotting (bottom panel.) Note that c-FLIP$_L$ was not detectable by immunoblot in this cell line.

2c. Recruitment of FADD to FAS is stimulated by cell detachment. MCF10a cells were treated with agonistic FAS antibody 2R2 under attached or suspended conditions for the indicated times, cells were lysed and the 2R2-FAS-DISC complexes were precipitated with protein A. The precipitates were analyzed for FAS and FADD on a Western blot. (In the lane labelled "-lys", lysate was omitted; hence the corresponding band in the other lanes is an antibody artifact.)

Figure 3. FADD is a nuclear protein in attached cells and is exported to the cytoplasm in detached cells.

3a. FADD is a nuclear protein in MCF10a cells. (*top panel*) MCF10a cells were analyzed for FADD localization by immunofluorescence and the image compared with a the location of the nucleus indicated by DAPI staining. (*middle panel).* Immunofluorescent staining was carried out after incubation of the primary antibody with recombinant maltose-binding protein fusion proteins of FADD or c-jun. (*lower panel).* MCF10a cells were infected with a retrovirus containing HA-tagged FADD and stained with anti-HA antibody.

3a (cont'd). (*upper two panels)* FADD is nuclear in other cell lines (HT1080: fibrosarcoma, HUVEC: human umbilical vein endothelial cells, Caco-2: colon carcinoma, HaCat: immortalized normal keratinocytes). (*bottom panel*) the TNFR adaptor protein TRADD is non-nuclear.

3b. FADD is exported in detached cells. Yellow fluorescent protein-FADD-nuclear localization signal expression constructs (YFP-FADD-NLS) containing wild-type FADD or mutant FADD sequences were transfected into MCF10a cells. Attached or 15-minute

detached cells treated in the presence or absence of 2R2 were then scored for nuclear (N) or nuclear plus cytoplasmic (NC) localization of the fluorescent tag; representative images used to score the cells are shown in the top panel. In the first series of bars, YFP-NLS was transfected as a control. The percentages of NC FADD in attached cells were subtracted from the values shown. (N.B., the fraction of cells undergoing nuclear export of FADD may have been underestimated due to interference by dimerization through YFP domains and/or failure to detect cells in which only low levels were exported.)

Figure 4. hmsn5 is a candidate FADD-shuttling protein.

4a. hmsn5 interacts with FADD.

(top left panel): GST-FADD expression constructs containing the FADD mutants indicated at the top of the panel were cotransfected with hmsn5-his expression constructs into 293T cells. Cell lysates were precipitated with glutathione-sepharose and the precipitates were analyzed on a Western blot using anti-tetra-histidine antibody (top row.) The middle and bottom rows demonstrate the presence of the hmsn5-his and GST-FADD proteins in the transfected cell lysates. *(top right panel): A* maltose binding protein-hmsn5 expression construct (mbp-hmsn5) was cotransfected with an HA-FADD expression construct into 293T cells, and lysates prepared in the presence of kinase and phosphatase inhibitors were precipitated with amylose resin. The precipitates (PD) were then analyzed for FADD using anti-HA antibodies; a dilution of the unselected lysate (IN) is shown. *(lower panel):* Recombinant hmsn5-his protein (purified from E. coli) was mixed with recombinant GST-FADD (wildtype) or GST-FADD (S194A) protein

(purified from transfected 293T cells) in the presence or absence of RanQ69L protein (purified from E. coli.) After incubation, glutathione beads were washed and analyzed for hmsn5-FADD interaction by Western blotting with anti-tetra-his antibody. GST-p130cas was used as a negative control (ctrl.)

4b. Crm1 does not have the properties of a FADD-exportin, and caspase activity is not required for export. *(top panels).* The effects of leptomycin B (LMB) and the pan-caspase inhibitor zVAD-fmk on FADD export (assayed as described above) are shown; this concentration of LMB effectively inhibited export of a green fluorescent protein-tagged HIV rev protein, and zVAD-fmk inhibited caspase activation to undetectable levels (data not shown.) *(bottom panel).* FADD did not associate with crm1: Gst-FADD or GST-PKI expression constructs were cotransfected with a crm1 expression construct into 293T cells and the glutathione-sepharose-precipitated protein was analyzed for crm1 on a Western blot.

4c. Export-deficient FADD mutants are also import-deficient. HA-FADD expression constructs containing the indicated FADD mutants were transfected into MCF10a cells, which were then immunostained with anti-HA antibody after being maintained in attached conditions. Representative HA-stained cells and the positions of their nuclei (DAPI) are shown.

4d. FADD S194A is a gain-of–function mutant with respect to toxicity. HT1080 or MCF10a cells were cotransfected with the indicated FADD mutants and a GFP expression vector. Cells were then fixed and DAPI stained, and the percentages of green

cells containing apoptotic nuclei were quantitated. Note that the percentages of apoptosis obtained may have been underestimated due to the loss of cells prior to fixation.

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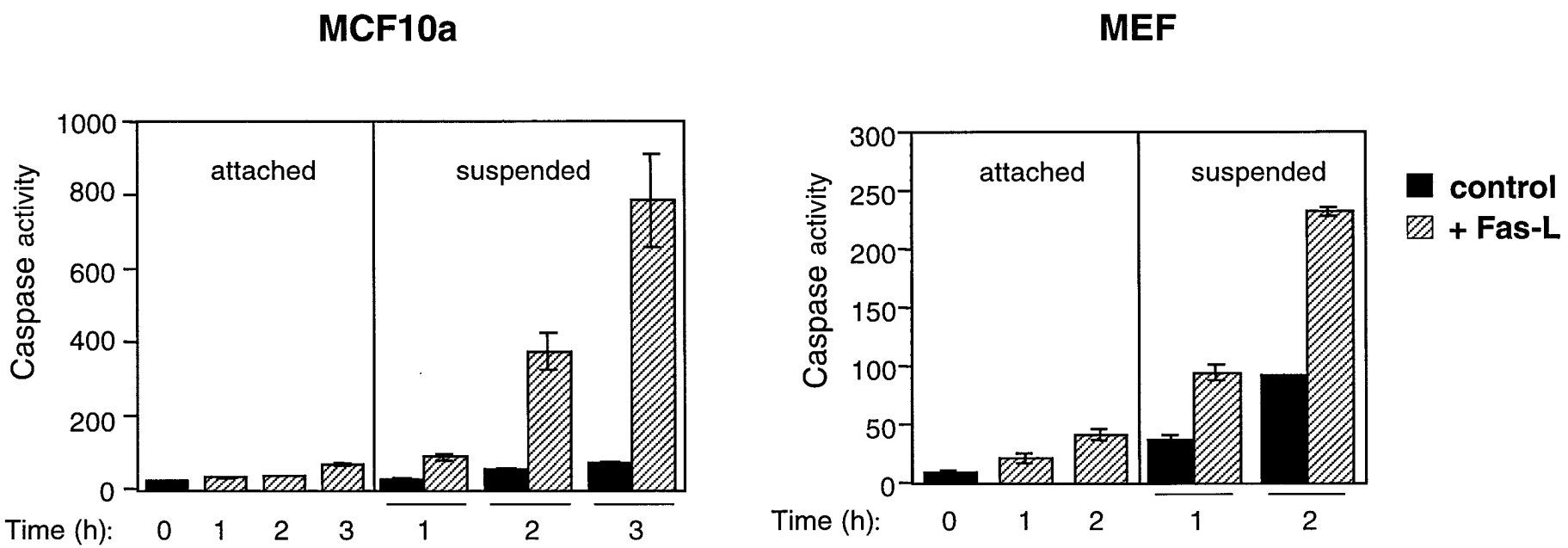
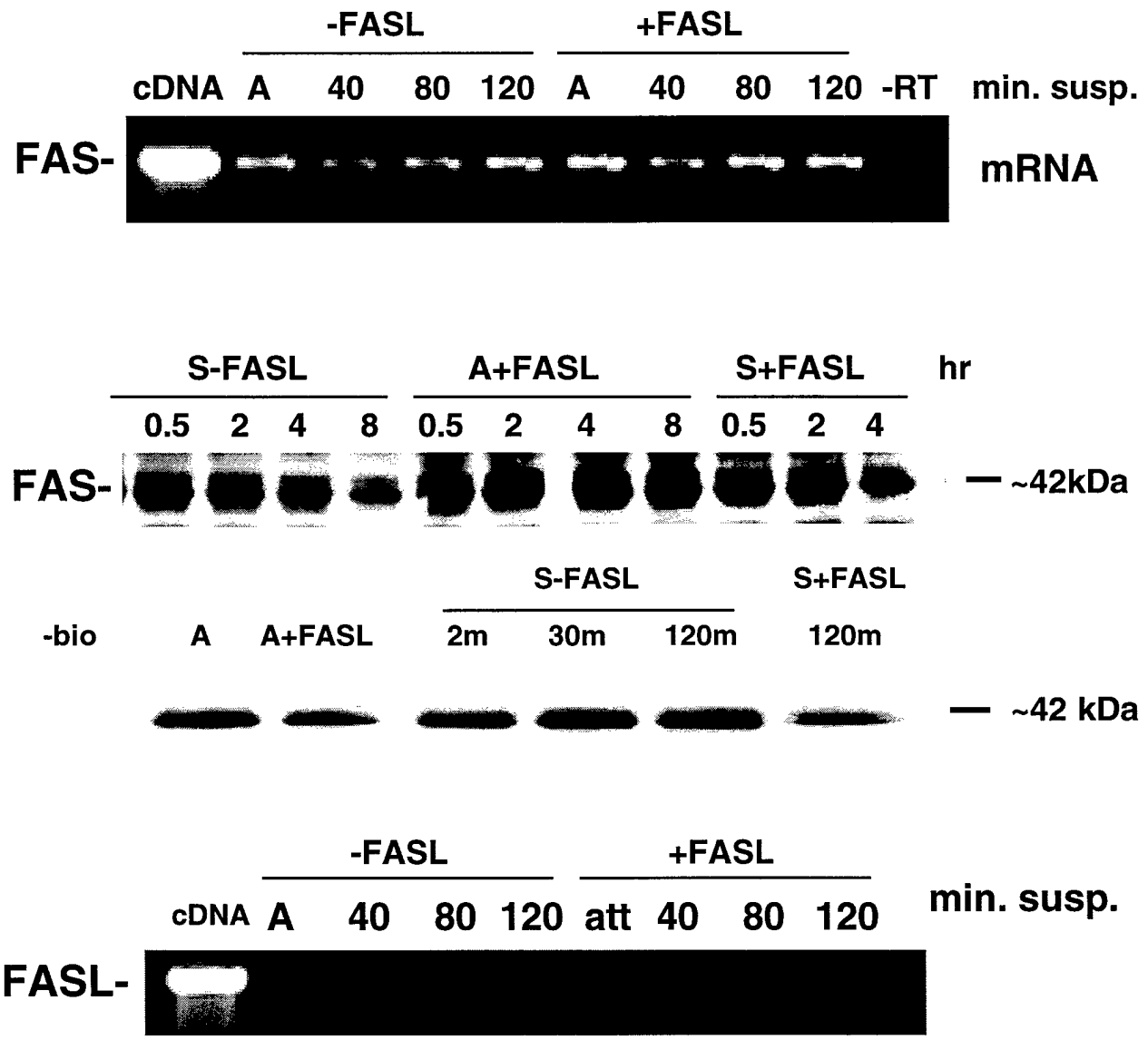

MCF10a
MEF
Caspase activity
1000
800
600
400
200
0
attached
suspended
Time (h):
0
1
2
3
1
2
3
300
250
200
150
100
50
0
attached
suspended
Time (h):
0
1
2
1
2
control
+ Fas-L

Figure 1a.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+FASL
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FAS-
mRNA
S-FASL
A+FASL
S+FASL
hr
0.5 2 4 8 0.5 2 4 8 0.5 2 4
FAS-
~42kDa
S-FASL
S+FASL
-bio A A+FASL 2m 30m 120m 120m
~42 kDa
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+FASL
cDNA A 40 80 120 att 40 80 120 min. susp.
FASL-

Figure 1b

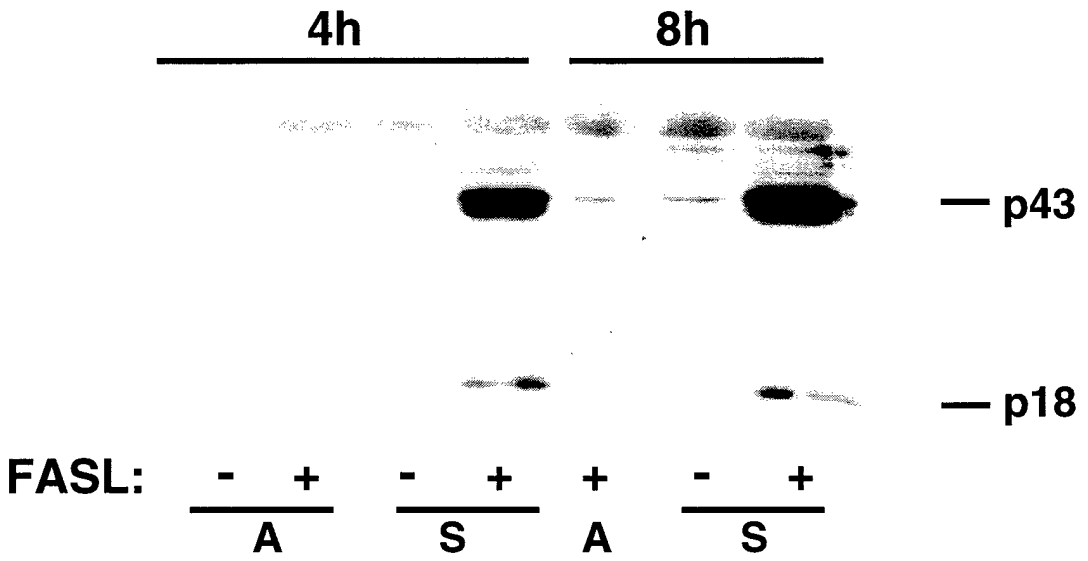
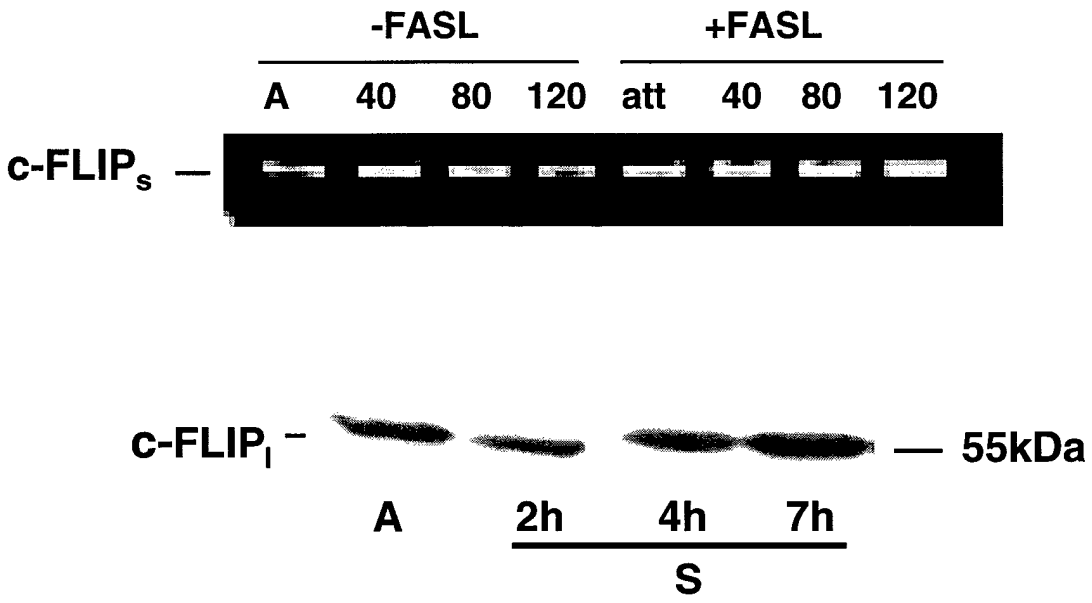


Figure 2a.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+FASL
A
40
80
120
att
40
80
120
c-FLIP$_s$
c-FLIP$_l$
55kDa
A
2h
4h
7h
S
S-FASL
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S+FASL
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2
4
2
4
0.5
2
4
FADD-

Figure 2b

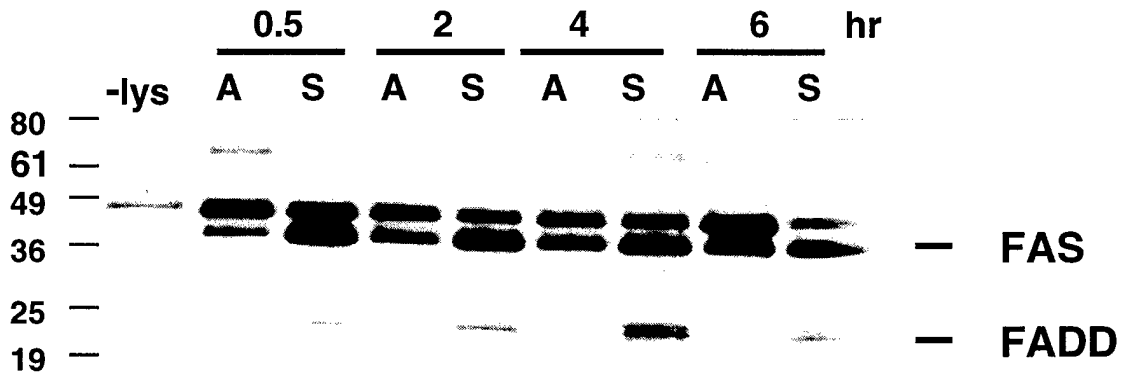
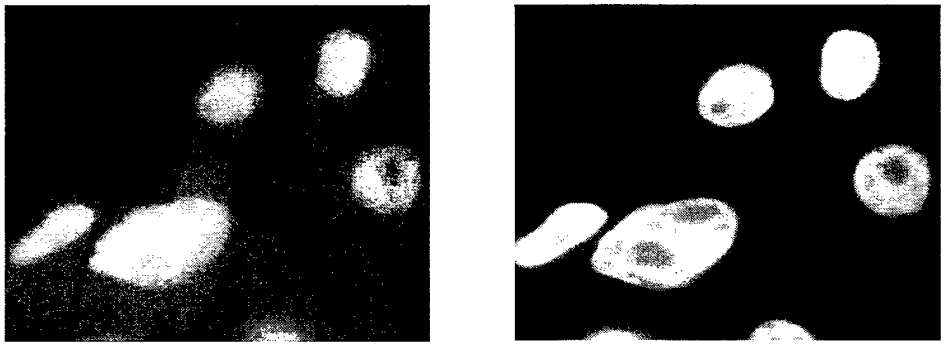
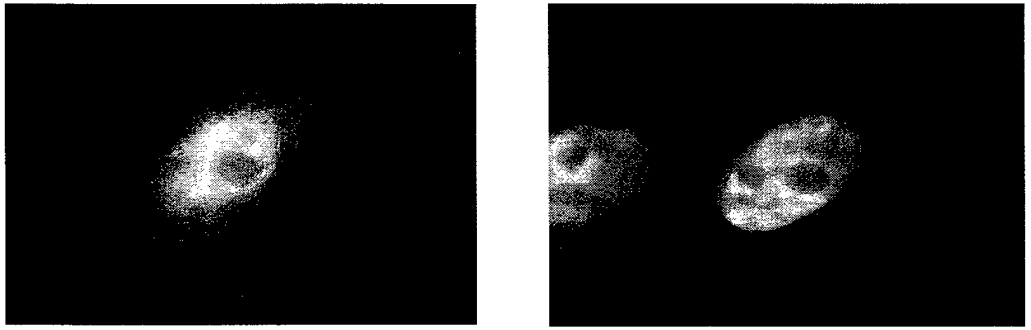


Figure 2c.



FADD DAPI

No Ag MBP-FADD MBP-Jun



HA DAPI

Figure 3a

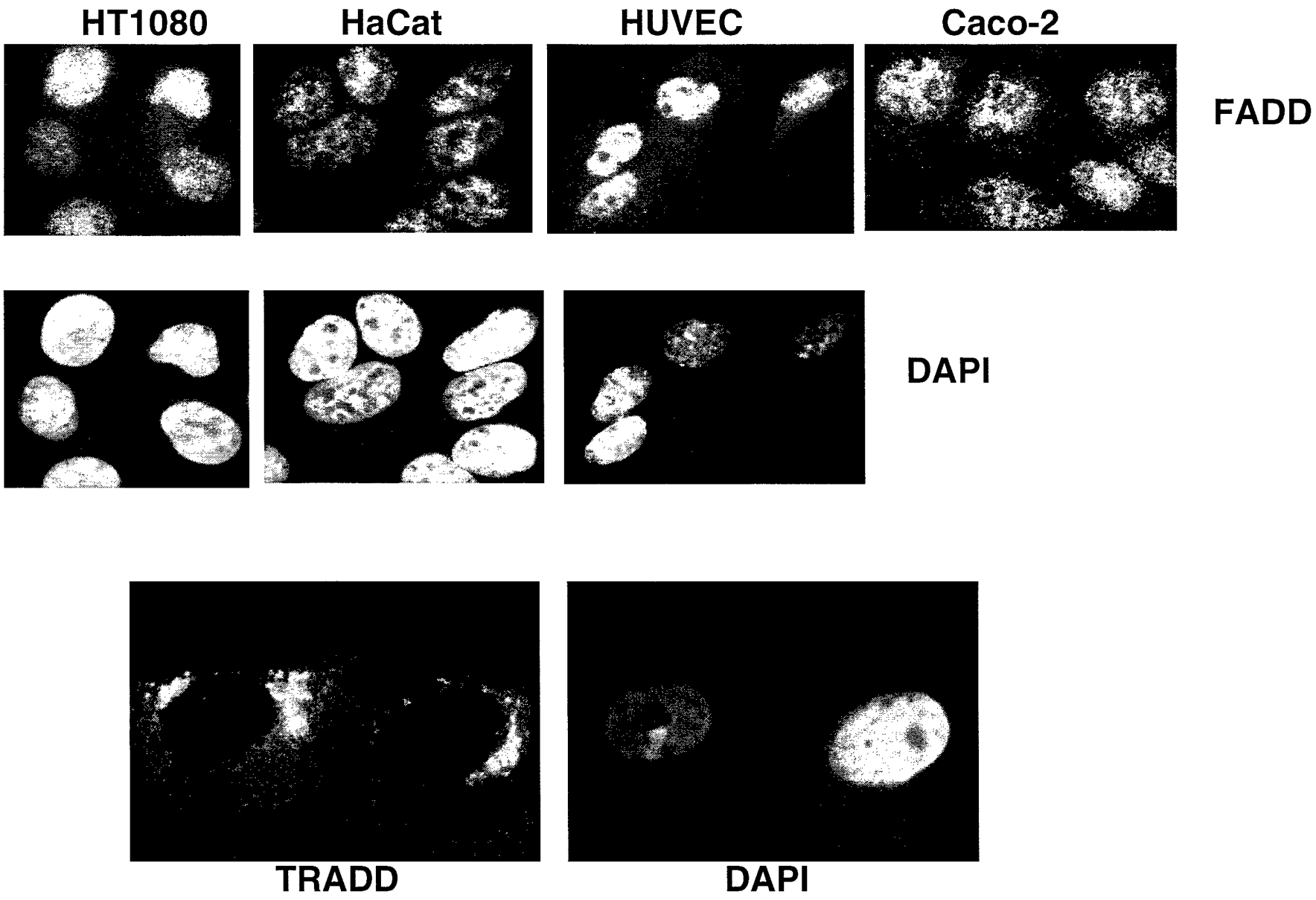
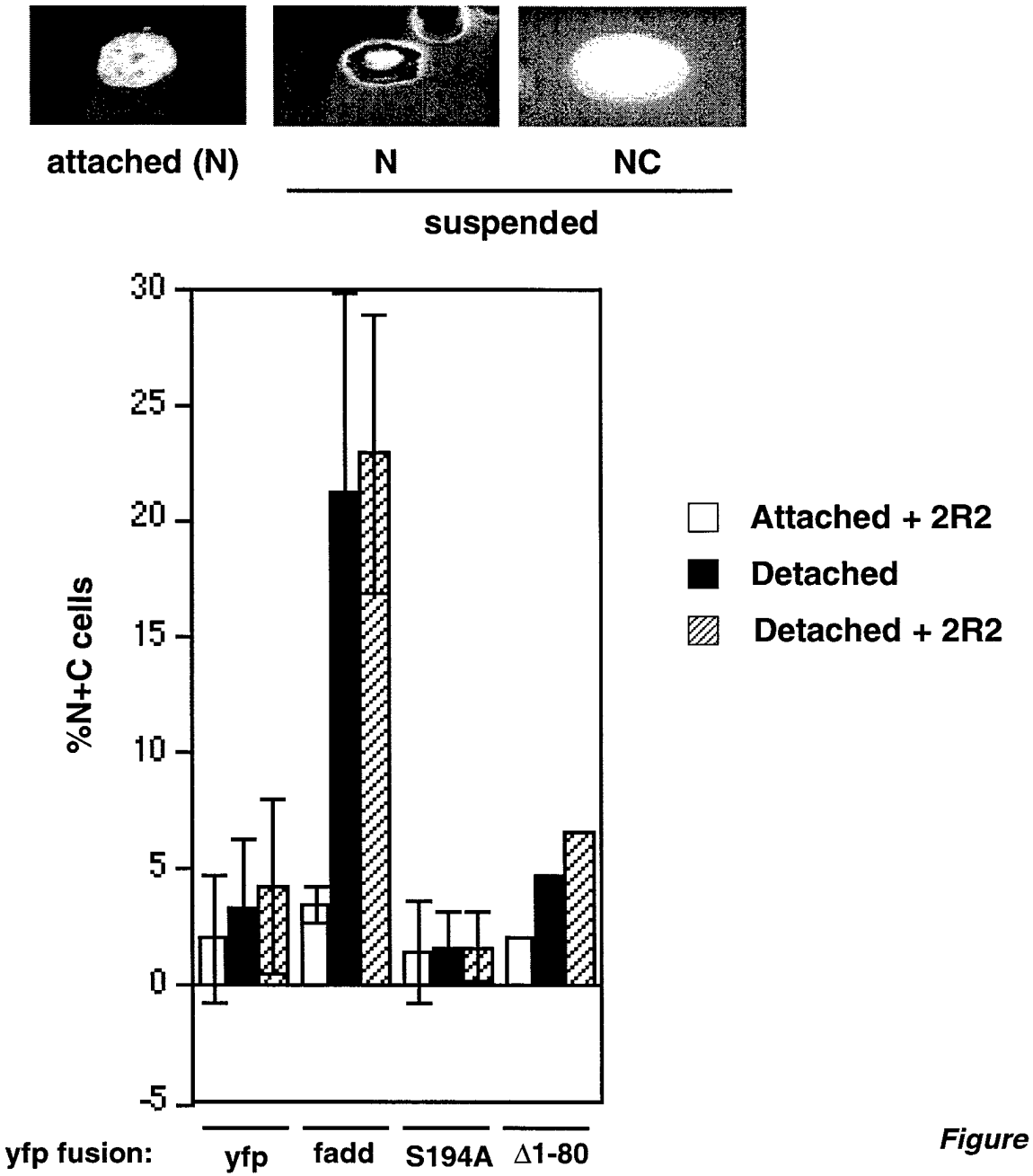


Figure 3a (cont'd)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NC
suspended
30
25
20
15
10
5
0
-5
%N+C cells
Attached + 2R2
Detached
Detached + 2R2
yfp fusion:
yfp
fadd
S194A
Δ1-80

Figure 3b.

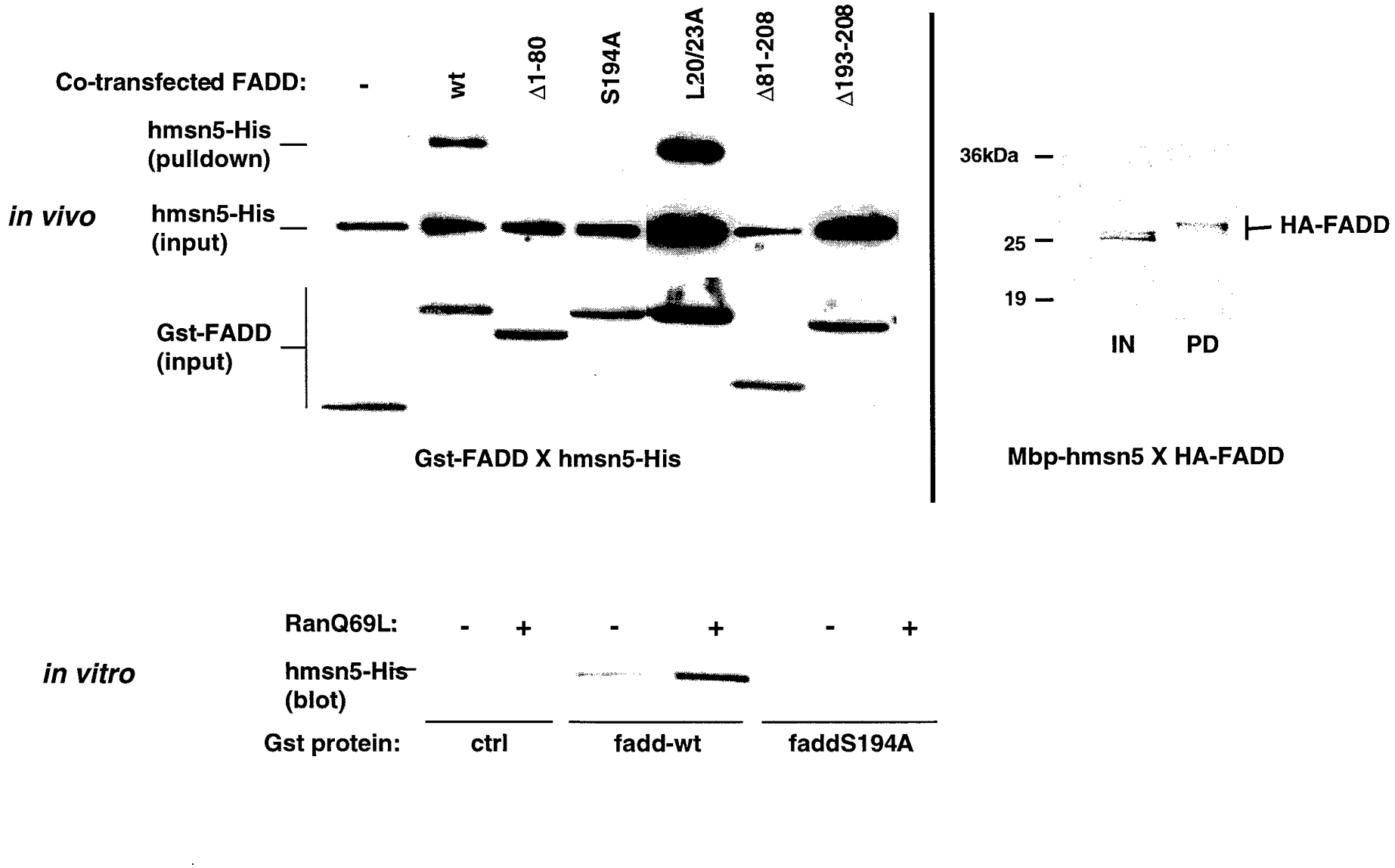
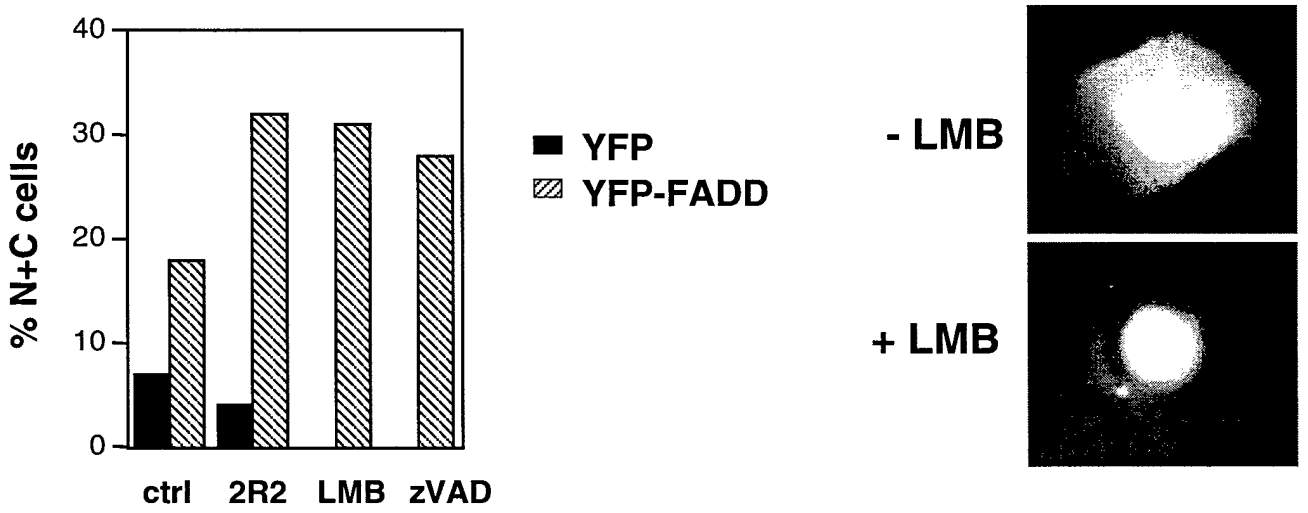

Co-transfected FADD:
-
wt
Δ1-80
S194A
L20/23A
Δ81-208
Δ193-208
hmsn5-His (pulldown)
in vivo
hmsn5-His (input)
Gst-FADD (input)
Gst-FADD X hmsn5-His
36kDa
25
19
HA-FADD
IN
PD
Mbp-hmsn5 X HA-FADD
RanQ69L:
- + - + - +
in vitro
hmsn5-His (blot)
Gst protein:
ctrl
fadd-wt
faddS194A

Figure 4a.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30
20
10
0
% N+C cells
ctrl
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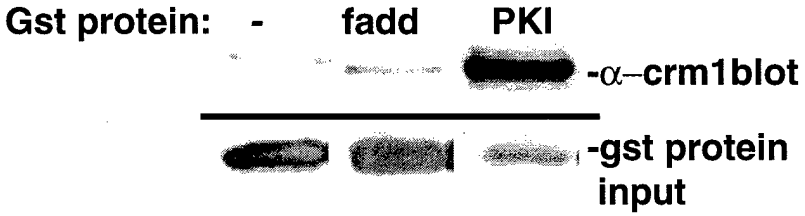
LMB
zVAD
YFP
YFP-FADD
- LMB
+ LMB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PKI
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input

Figure 4b.

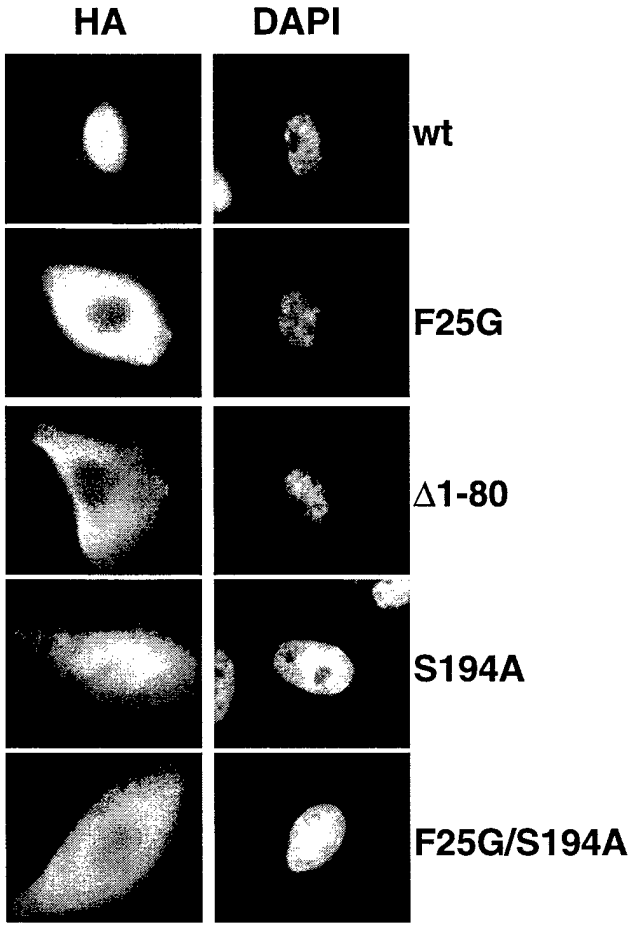
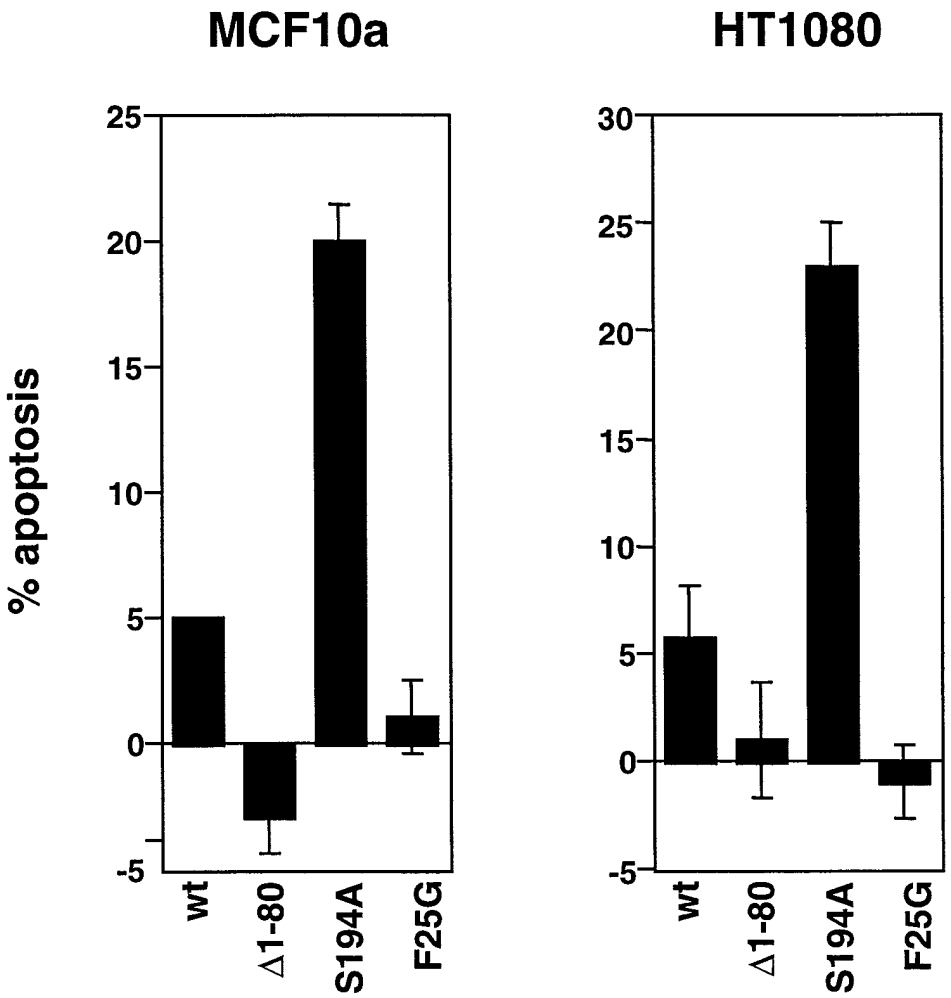


Figure 4c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HT1080
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15
10
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-5
30
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20
15
10
5
0
-5
wt
Δ1-80
S194A
F25G

Figure 4d